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# IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: IZHAR

Application No.: 09/411,863

Filing Date: October 4, 1999

For: EXOGENIC ALLELISM

Group Art Unit: 1638

Examiner: A. Kubelik

Attorney Docket No.: 85189-4300
Formerly: 00/20907

## DECLARATION OF DR. VERED YESODI UNDER 37 C.F.R. § 1.132

Assistant Commissioner of Patents
Washington, D.C. 20231

Sir:

I, Dr. Vered Yesodi, declare that:

1. I am a citizen of Israel and reside at 54 Irusim Street, Reut, ISRAEL.

2. I hold a Ph.D. in Genetics from The Hebrew University of Jerusalem, a M.Sc. degree from Tel-Aviv University, Faculty of Life Sciences, and a B.Sc. from Ben-Gurion University, Department of Biochemistry.

3. I am presently CEO and R&D Manager of Fertiseeds Ltd. Prior to this I was the Research Director of In-Vitro Fertilization (IVF) Laboratory, Racine IVF Unit at LIS Maternity Hospital in Tel-Aviv. I also did a Post doctorate at Tel-Aviv University, Medical School, Department of Embryology and was a research assistant at Volcani Center for Agricultural Research in the Department of Virology for 2 years. I have published nine scientific articles in highly regarded journals and books.

4. I have reviewed and understand the above-identified patent application, the newly amended claims and the Office Action, in particular the Examiner's rejections under 35 U.S.C. §112, first paragraph. I am making the following statements as one of ordinary skill in the art in support of the patentability of the present claims.

5. The above-identified application is directed to methods of generating exogenic allelism in a plant. The application is further directed to plants and plant seeds produced by these methods.

6. This declaration with accompanying PowerPoint presentation are provided to declare and set forth evidence that the disclosed methods are fully enabled and described in the specification. The PowerPoint presentation provides a detailed explanation of each step of the claimed methods. Evidence of the successful implementation of each step is also included. Furthermore, evidence of the actual generation of plants characterized by exogenic allelism using the claimed method is provided in Figure 13. Figure 13 provides the results of a gDNA analysis of 16 plants generated from the claimed method. All 16 plants were characterized by exogenic allelism.

7. The presentation describes two methods for generating exogenic allelism in plants: a method according to claim 47, and a method according to claim 49, both summarized for you convenience in Figures 1 and 2.

8. A model system of the presently claimed method was established in *Arabidopsis* plants. The model system employs two reporter genes, CFP (Cyan Fluorescent Protein) and GFP (Green Fluorescent Protein), as examples of the two exogenes. Both genes are under the control of the constitutive 35S promoter. Figure 3 shows the DNA constructs, plants and methods used and manipulated in the model system. The slides that follow Figure 3 provide detailed explanations of the steps with evidence of the successful implementation of each step, ultimately resulting in the production of plants having exogenic allelism.

9. For your convenience I provide the following brief explanation of the steps of the invention and point out the figures that correspond to each step. For a more detailed explanation please refer to the PowerPoint presentation.

- Step 1 is the generation of plants carrying a single copy of the expression cassette. Figures 4 and 5 illustrate the implementation of step 1.
- Step 2 of the model is the generation of plants carrying an active recombinase 1 and plants that carry a different recombinase 2. Figure 6 is a Western blot illustrating the successful execution of step 2. Figure 6 shows the expression of Cre recombinase and the DNA vector carrying the Cre-recombinase in the plant sample.

•Step 3 is the excision of one transgene from the expression cassette. Figures 8 and 9 correspond to Step 3 and provide a gDNA analysis of plants resulting from Step 3.

Step 4 is obtaining plants devoid of recombinase gene. Figures 10 and 11 show the selection of plants carrying the expression cassette, which are devoid of the recombinase gene.

•Step 5 is the crossing of homozygous-plants resulting from steps 1 and 4. Figure 13 corresponds with Step 5. Figure 13 is a gDNA analysis of 16 plants. The analysis demonstrated that 100% of the plants tested had exogenic allelism.

•Step 6 is the crossing of plants produced from step 5 (exogenic allelism) with wild type *Arabidopsis* plants. Figure 14 is a gDNA analysis of the progeny of step 6 showing that 100% of the exogenic alleles segregated, and table 1 presents the statistical distribution of alleles in 62 plant pf this progeny. Figure 15 summarizes the concept of exogenic allelism, showing that cross of plants from step 5 with wild type *Arabidopsis* plant will result in 100% segregation of the exogenic alleles.

10. As one skilled in the art, based on my review of the application and evidence in the PowerPoint presentation, it is my opinion and judgment that the invention as presently claimed in claims 47, 49, 51 and 56-79 is enabled, and that one of ordinary skill in the art would clearly understand this from a review of the same. After reviewing the originally filed application I reasonably conclude that the inventors were in possession of the invention as presently claimed at the time of filing, and that this is evident to any skilled artisan.

11. I further declare that all statements made herein of my knowledge are true and all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing thereon.

Dated: 12/03/2003

Vered Yesodi

**Vered Yesodi, Ph.D.**

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# "Exogenic Allelism"

Power-Point Presentation for U.S Patent Application No. 09/411,863, filed October 4. 1999

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**Figure 1**

# Claim 47

a. Providing a first plant and a second plant each including an expression cassette in the same chromosomal location.....

Pro1 - SSR1 - **Pro2+Allele II** - SSR1 –Allele I

b. Introducing a recombinase into said first plant, so as to excise said third segment thereby operatively adjoining said first transcribable polynucleotide sequence to said first promoter sequence.

**Introducing** Pro-recombinase

**Pro1**-SSR1-**Allele I**

c. Selfing a plant resulting from step (b) and selecting a progeny which is devoid of said recombinase.

Analysis for recombination
↓
Self pollination
↓
**Devoid of recombinase**

Cross

d. Crossing a plant resulting from step (c) with said second plant thereby obtaining an offspring characterized by **exogenic allelism**.

Pro1-SSR1-**Pro2+Allele II**-SSR1 –Allele I

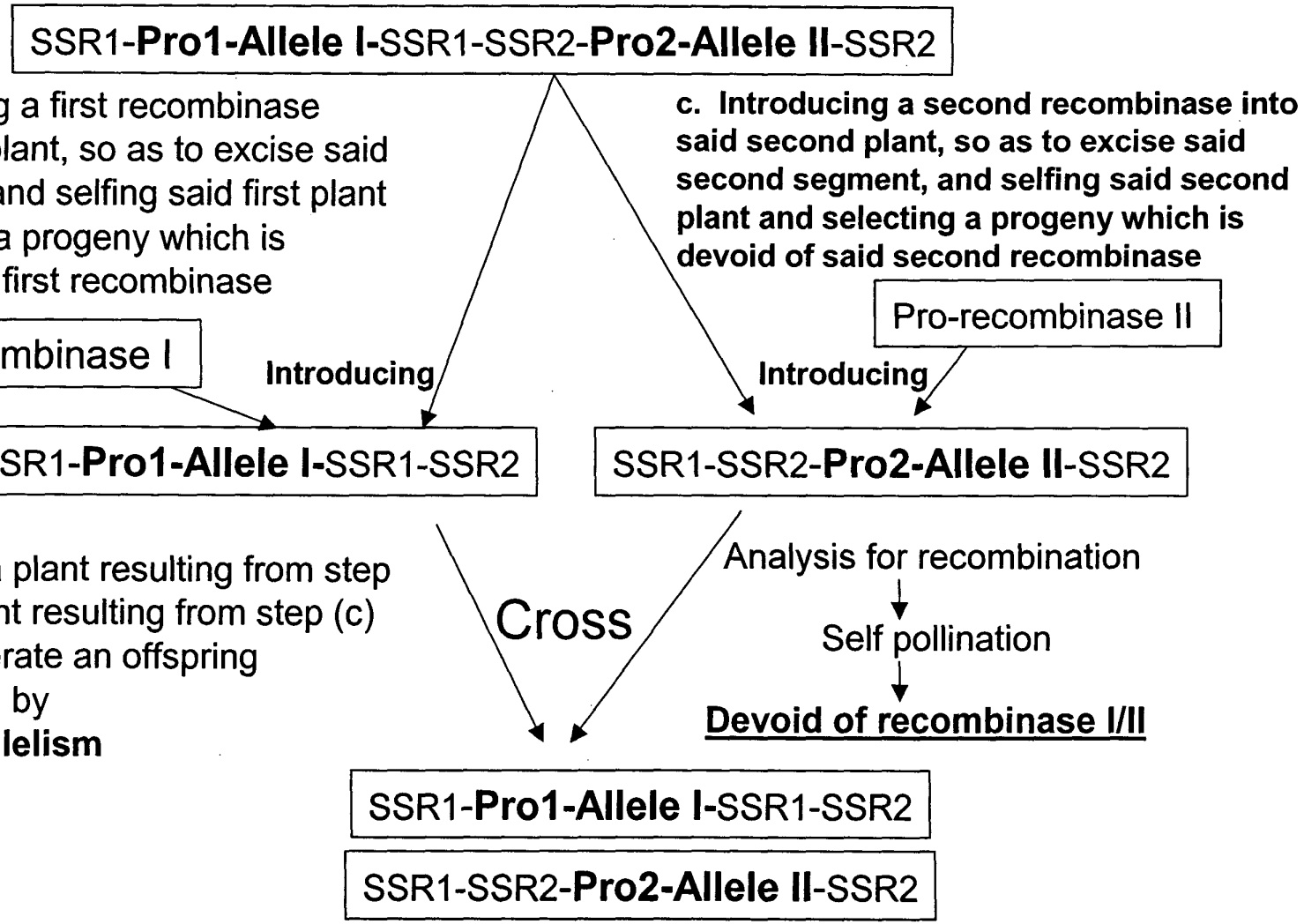
**Pro1**-SSR1-**Allele I**

*Abbreviation*: Pro = Promoter; SSR = Site Specific Recombination

**Figure 2**

# Claim 49

a. Providing a first plant and a second plant each including an expression cassette in the same chromosomal location.....



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# A model system for Exogenic Allelism

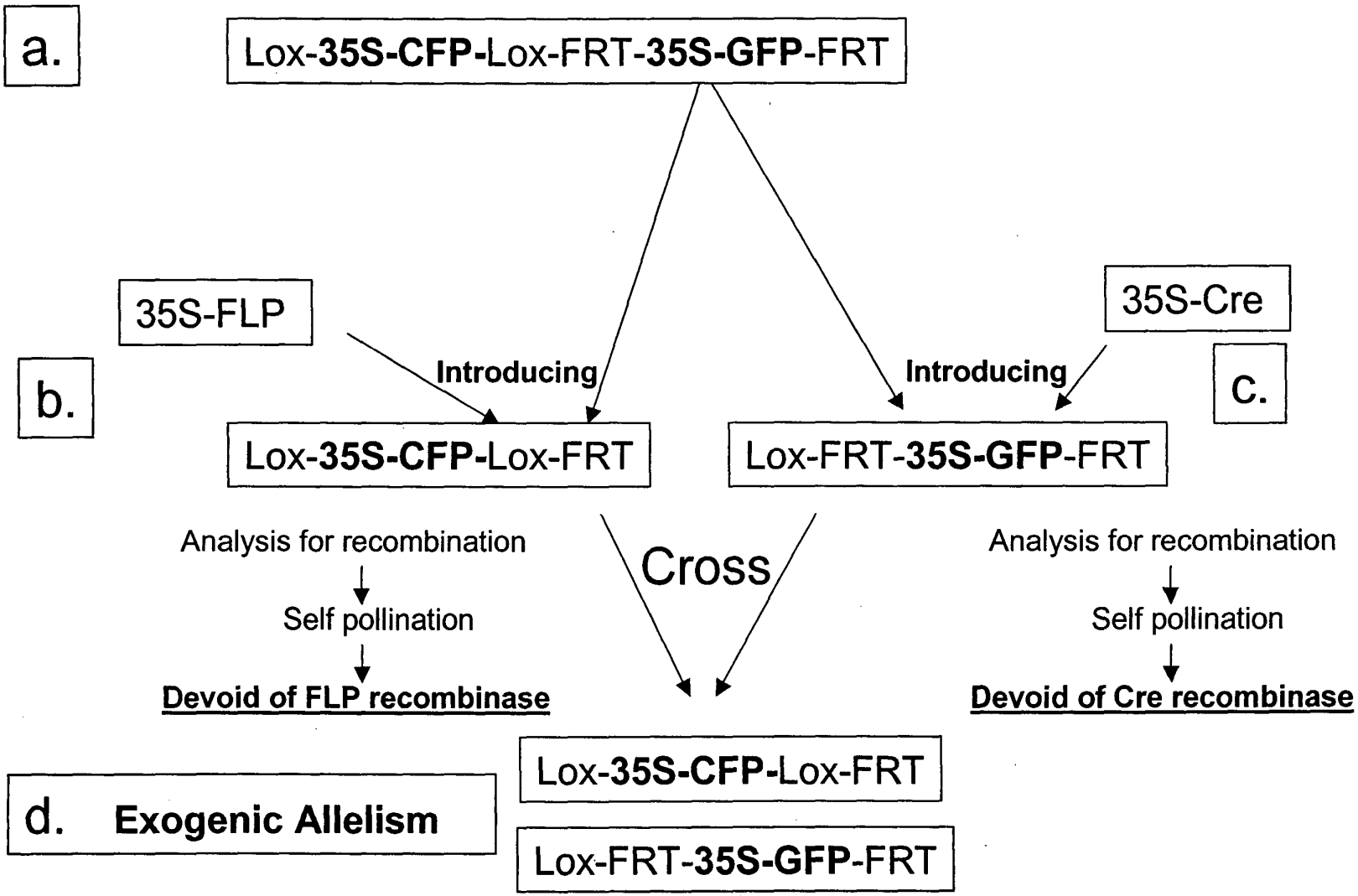
We established a model system in *Arabidopsis* plants to demonstrate proof of concept for Exogenic Allelism

The model system uses two reporter genes as example of the two exogenes: CFP (Cyan Fluorescent Protein) and GFP (Green Fluorescent Protein). Both genes are under the control of the constitutive 35S promoter.

Figure 3 outline the DNA constructs, plants and methods used in the model system.
Lox is the recognition DNA sites for Cre recombinase; FRT is the recognition site for FLP recombinase.

**Figure 3**

# A model system for Exogenic Allelism



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## Following detailed steps and examples show the generation of plants exhibiting Exogenic Allelism

### Step 1

Generation of plants which carry a single copy of the expression cassette, where transgene 1 with its promoter (35S::CFP), flanked by site specific recombination sequences (S.S.R.1.; e.g. Lox), is adjacent to transgene 2 with its promoter (35S::GFP) flanked by a different recombination sequences (S.S.R.2.; e.g. FRT).

Forty-two transformed *Arabidopsis* plants were generated on hygromycin. Ten of these plants showed strong phenotypic expression (fluorescence of GFP and CFP) and a few were found to carry a single copy of the expression cassette.

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**Figure 4** illustrates the DNA construct and the endonuclease restriction map of the integrated DNA. The right panel shows that the transgenic *Arabidopsis* plants integrated the proper DNA construct.

**Figure 5** upper panel shows the expression of both CFP and GFP in transgenic *Arabidopsis* plants. This illustrates the ability to choose plant with integrated expressible polynucleotide.

**Figure 5** lower panel shows the selection of plants carrying a single copy transgene. Several transgenic Arabidopsis plants carry only one copy of the expression cassette.

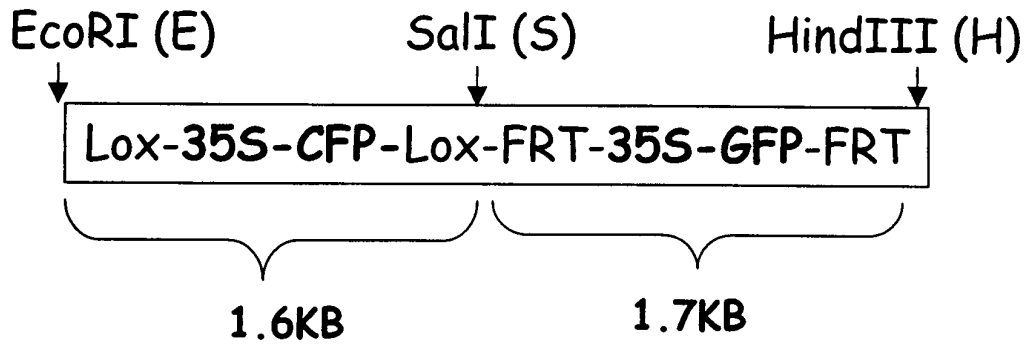
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**Figure 4**

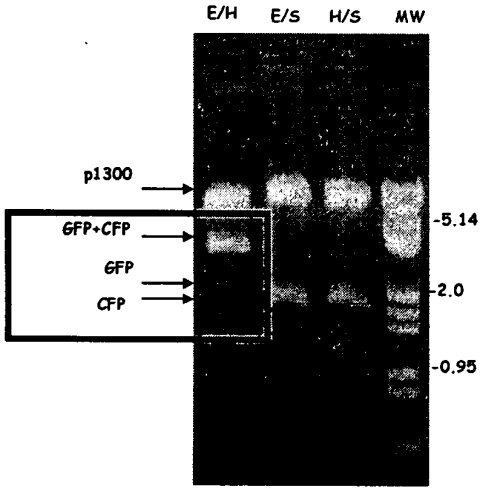
# Claim 49a

"Providing a first plant and a second plant each including an expression cassette in the same chromosomal location"

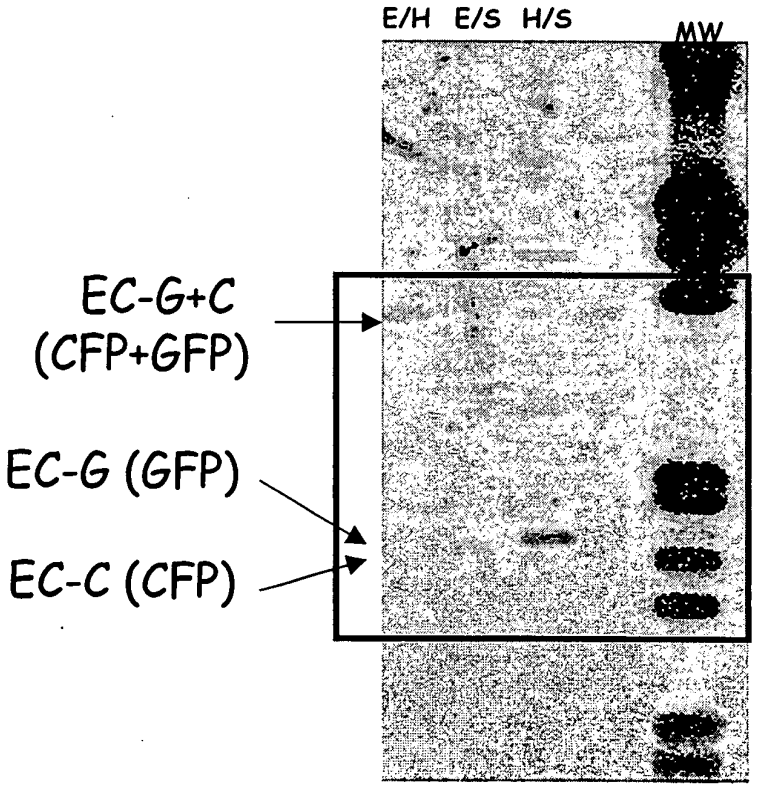
## Expression cassette construct




Plasmid DNA




Transgenic Plant (Southern blot)



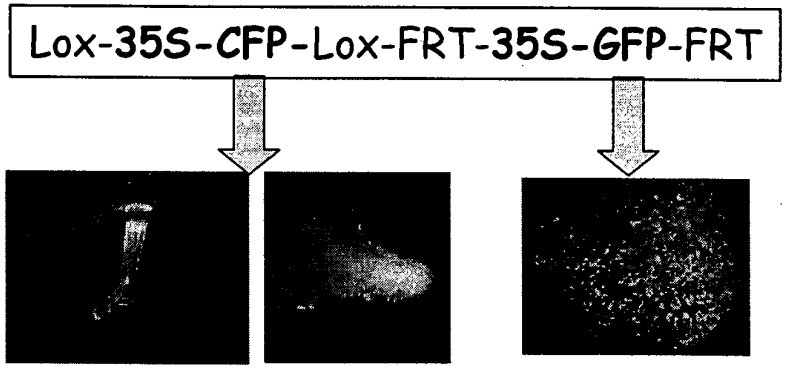
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**Figure 5**

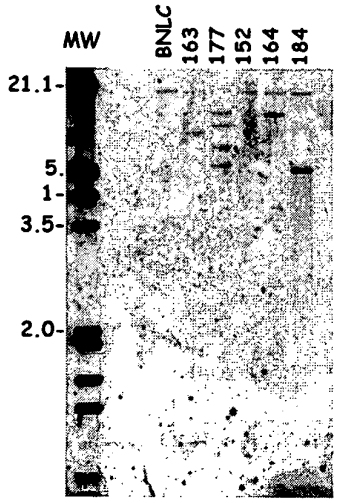
# Claim 49a (continuation)

"Providing a first plant and a second plant each including an expression cassette in the same chromosomal location"

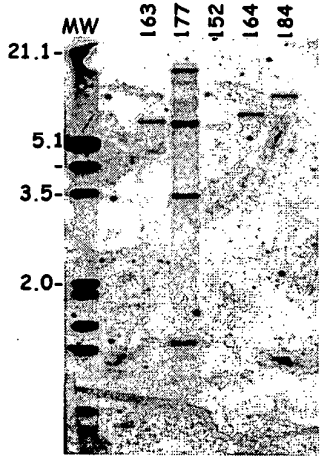
## Phenotypic analysis of transgenic *Arabidopsis* plant




## Copy number of Expression Cassette in transformed *Arabidopsis* plants




EcoRI




HindIII

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**Step 2** – Generation of plants which carry an active recombinase 1 (e.g. Cre) and plants which carry a second and different recombinase (e.g. FLP).

25% of the Cre-transformed *Arabidopsis* plants showed expression of the Cre recombinase. Three plants carry a single copy. Cre protein was found in several transformed *Arabidopsis* plants. Several of these transformed plants showed Cre activity.

Forty transformed plants carrying the FLP construct were generated. Due to lack of FLP antibodies, analysis of protein expression could not be made. Northern blot analysis is underway. The activity of random 3 FLP-transformed plants was studied. Out of these plants, non were found to be active in *Arabidopsis*

**Figure 6** demonstrates the introduction of recombinase according to the present system. It shows the expression of Cre recombinase (Western Blot) and the DNA vector carrying the Cre-recombinase

**Figure 7** summarizes steps 1 and 2

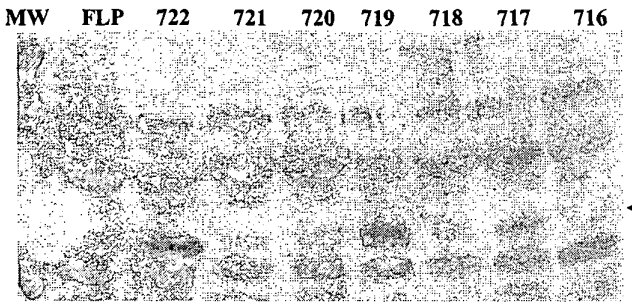
**Figure 6**

# Claims 47b and 49b and c

## "introducing a recombinase ....."

1. Sexual crosses with plants which carry an active recombinase (Cre+ or FLP+)

Plants expressing Cre recombinase




First generation

← Cre protein →



Third generation

2. Second transformation with DNA that carry expressible recombinase (Cre+ or FLP+) gene

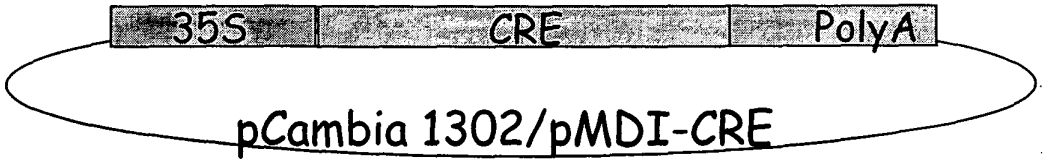
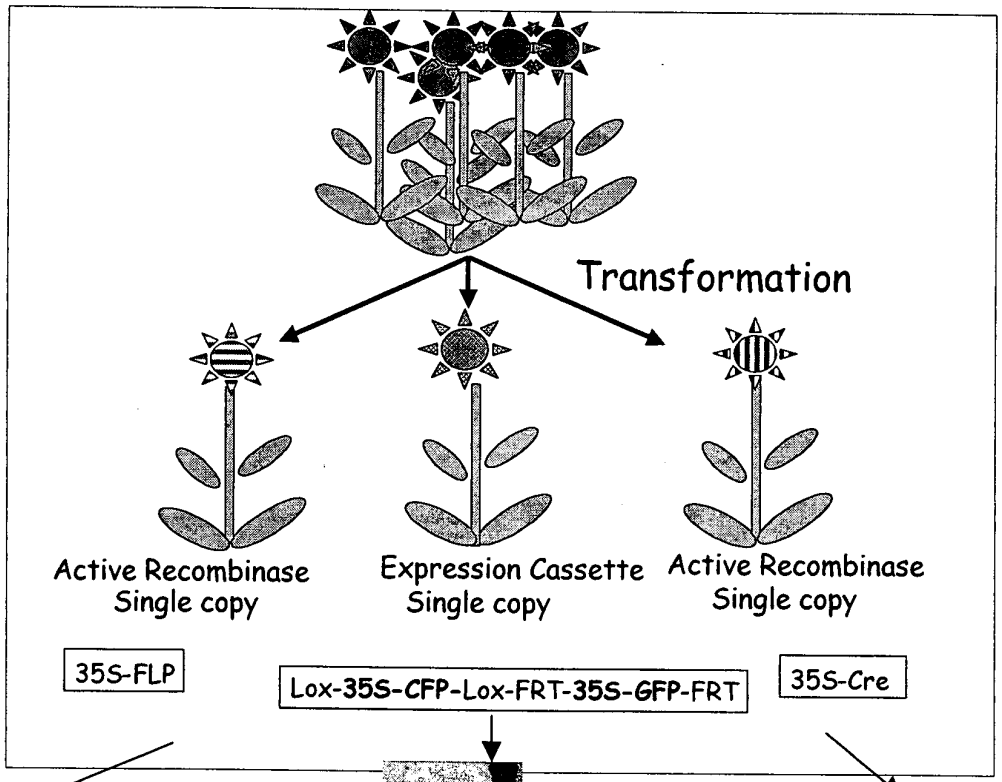


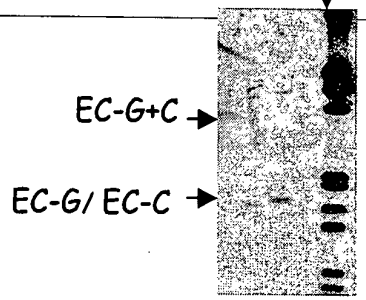
Figure 7

# Claim 49a, b and c - summary

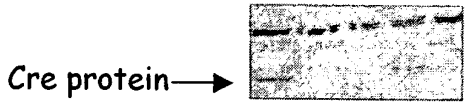



Under analysis

Plants which carry an activ FLP recombinase (claim 49b)




Plants which carry single copy of the Expression Cassette (claim 49a)




Plants which carry an active Cre recombinase (claim 49c)

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**Step 3** - Excision of one transgene from the expression Cassette.

Several methods can be used to introduce a recombinase gene (Cre or FLP) into the transformed plants that carry a single copy of the expression cassette.

We have used the following two methods:

1. Crosses between transformed plants carrying the expression cassette (Figure 5) and plants that carry an active recombinase (Figure 6).

2. Introducing a DNA that carries expressible Cre and/or FLP recombinase (Figure 6) into a plant carrying the expression cassette (Figure 5).

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The cross between transgenic *Arabidopsis* plants containing the expression cassette (Figure 5) and transgenic *Arabidopsis* plants expressing active Cre- recombinase (Figure 6) yielded many seeds. Due to time considerations, we used a heterozygote plant carrying active Cre-recombinase for further crosses. As a result only part of the progeny, that is plants that received an active Cre-recombinase gene, showed excision.

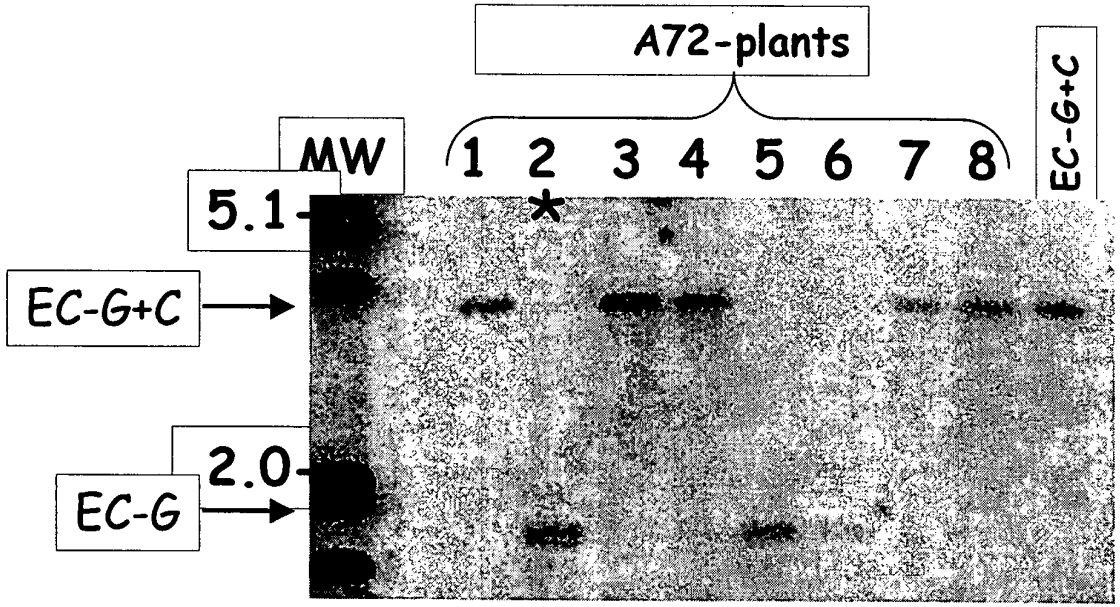
**Figure 8** shows analysis of genomic DNA (Southern Blot) isolated from the progeny resulted from the crosses described above. Genomic DNA was digested with EcoRI/HindIII endonuclease, separated over 1% agarose gel, transferred to nylon membrane and hybridized to DIG labeled GFP (according to manufacturers instruction). Band at about 3.2 kb indicates the entire expression cassette (CFP+GFP) while band at 1.7 kb indicates the presence of GFP allele alone, e.g., excision of the entire DNA between the Lox sites (see Figure 4)

**Figure 8**

# Claim 49c and 47b

"Introducing a recombinase (first method)....so as to excise said first/third segment ....."

DNA analysis of plants resulting from cross between EC-G+C and Cre + plants (T1)




Plants that showed excision (EC-G) received the active Cre-recombinase gene. Plants that contained the entire cassette (both alleles, EC-G+C) did not receive the Cre-recombinase gene (data not show)

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Plants which carry single copy of the expression cassette were subjected to a second transformation. Seeds were germinated on double antibiotics and plants were analyzed for excision event. Southern blot analysis of T1 plants showed excision of the transgene that was flanked by Lox sites when Cre-recombinase gene was present.

Using the same method, FLP-recombinase was not active in these plants.

**Figure 9** shows an example of one plant from the above described progeny, that is the result of excision after second transformation. Analysis was preformed as describe for Figure 8 above.

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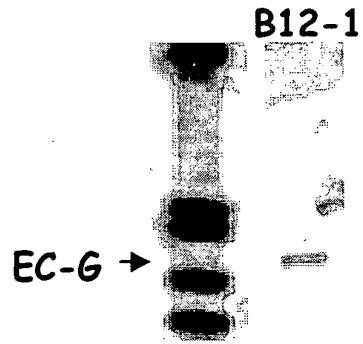
**Figure 9**

# Claim 47b, 49c

"Introducing a recombinase (second method)....so as to excise said first/third segment ....."

DNA analysis of plants resulting from second transformation of EC-G+C plants with Cre-expressing vector (T1)

**Transformation of plants which carry the Expression Cassette (EC-C+G) with DNA that carries expressible Cre recombinase (Figure 6)**



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**Step 4** - Obtaining plants devoid of the recombinase gene.

The progeny resulted from self-pollination of the T1 plants (from step 3, Figures 8 and 9) will segregate in a typical Mendelian manner (9:3:3:1). Therefore, out of 16 (T2) plants 3 will carry the expression cassette (after excision) only, and not the recombinase genes.

Southern blot analysis of T2 plants detected at list one plant that contained the expression cassette after excision (by Cre-recombinase) and not the recombinase gene. This was found to be the same using both methods for introducing the recombinase.

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**Figure 10** shows genomic DNA (gDNA) analysis of T2 plants following introduction of recombinase by the cross method. Analysis was done as describe in Figure 8. **Plant A72-2-5 (EC-G) carries only GFP and not Cre recombinase.**

**Figure 11** shows gDNA analysis of T2 progeny plants following introduction of recombinase by the second transformation method. Analysis was done as describe in Figure 8. **Plant B12-1-5 (EC-G) carries only GFP and not Cre recombinase.**

Self-pollination of these plants will enable selection of plants that are homozygous for each allele.

These plants were used as the parental line for the production of the Exogenic Allelism.

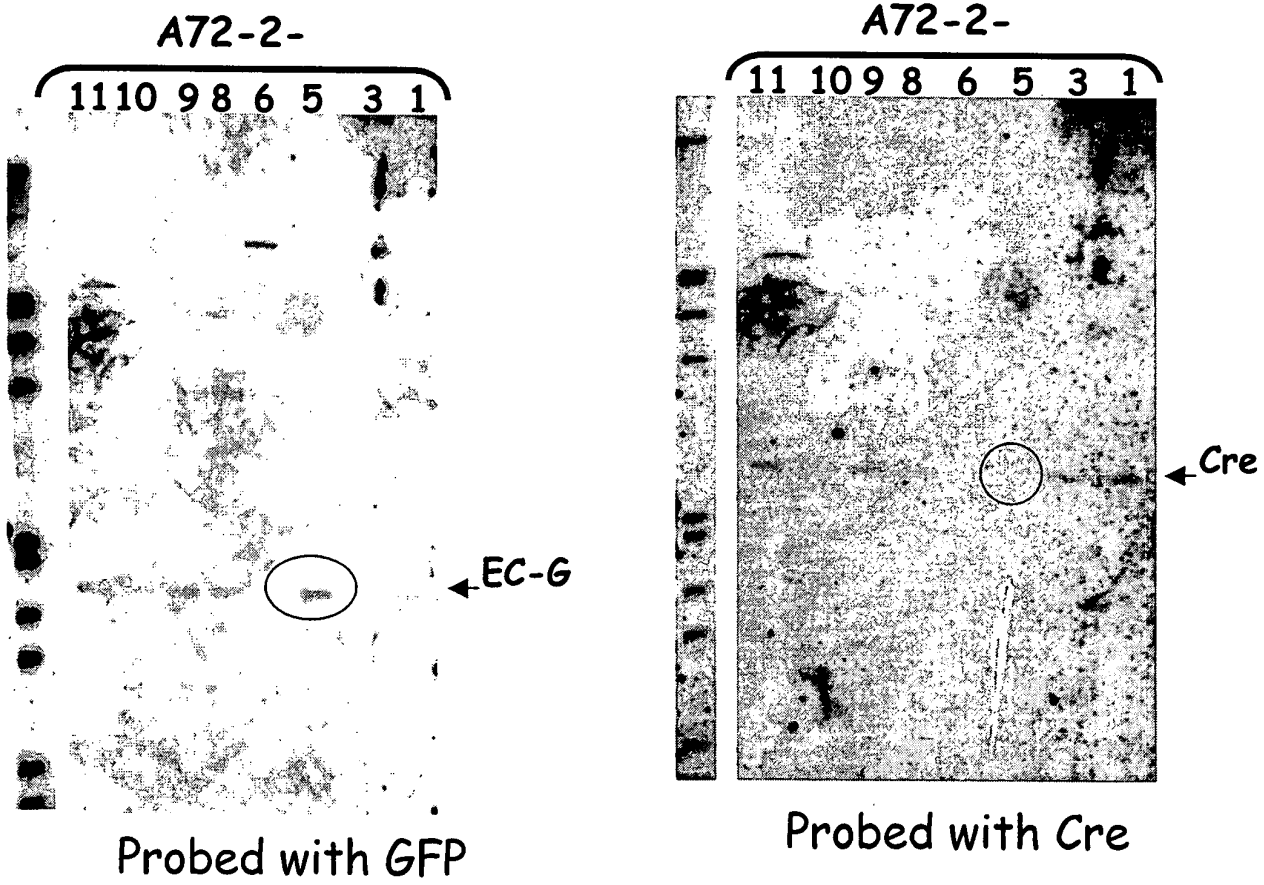
**Figure 10**

# Claims 47c, 49c

"selfing a plant resulting from step (b) and selecting a progeny devoid of said recombinase having the expression cassette wherein the third segment has been excised"

**DNA analysis of plants following self pollination (T2) (cross A72-2)**

A72-2-5 = Only GFP (**EC-G**), devoid of Cre recombinase




**Plant A72-2-5: excision event occurred, and the plant is devoid of Cre recombinase**

**Figure 11**

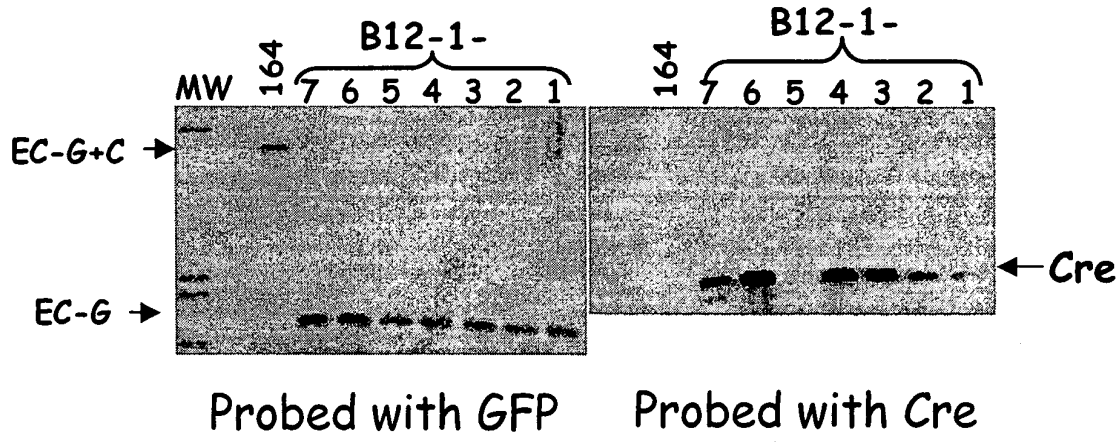
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# Claims 47c and 49c

"selfing a plant resulting from step (b) and selecting a progeny devoid of said recombinase having the expression cassette wherein the third segment has been excised"

**DNA analysis of plants following self pollination (second generation)**

**B12-1-5 = Only GFP (EC-G), devoid of Cre recombinase**



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Due to technical problems, we were unable to isolate *Arabidopsis* transformed plants that show excision by the FLP recombinase

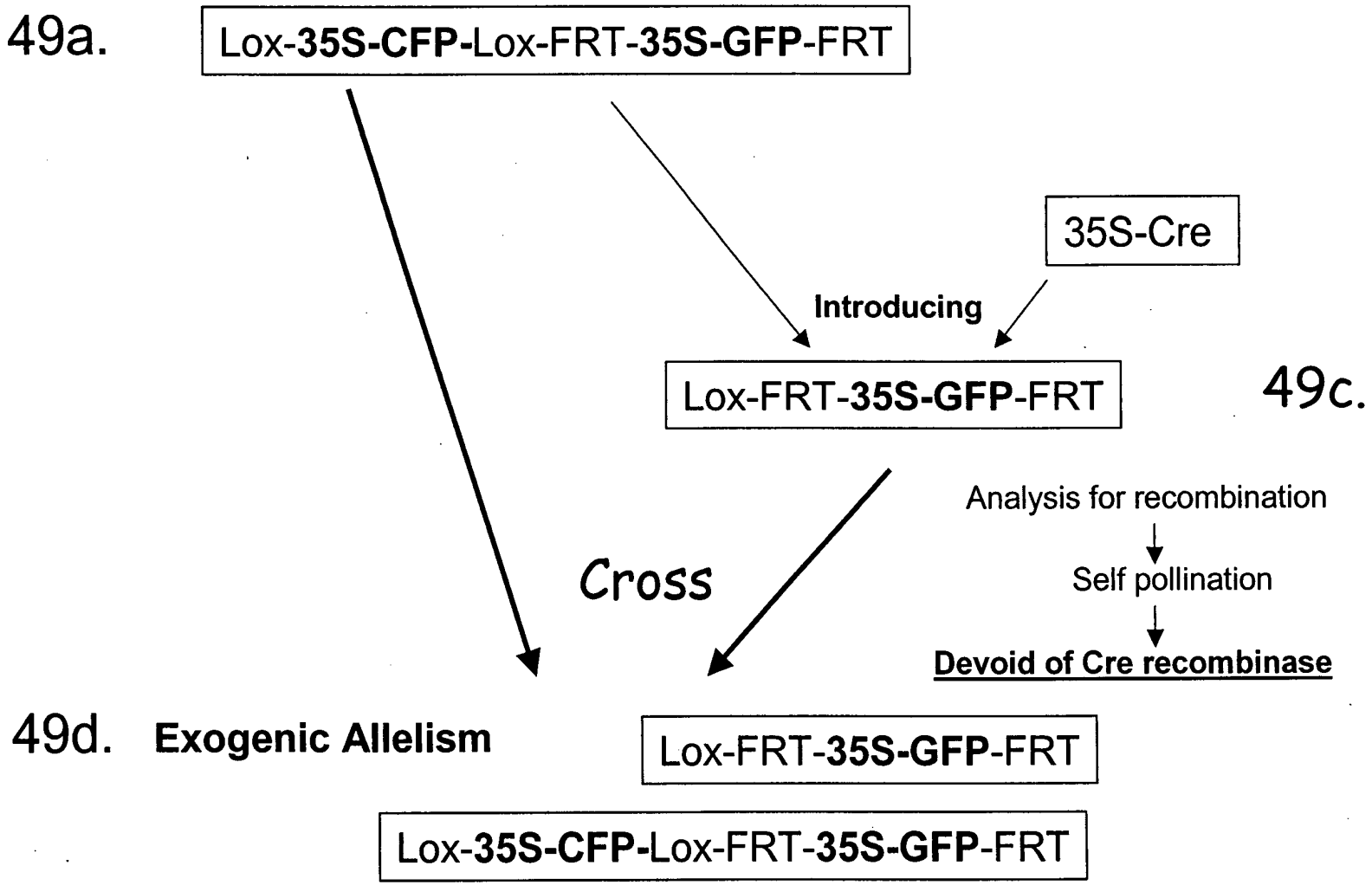
Additional experiments are done in order to isolate *Arabidopsis* plants that have FLP recombinase activity.

In parallel, we are studying the ability of FLP recombinase to work in tomato plants.

In order to proof enablement of the patent concept and obtain plants that are characterized by Exogenic Allelism, we created a modified model as outline in Figure 12.

**Figure 12**

A modified model for Exogenic Allelism using plants according to claim 49a and 49c as the two exogenes



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**Step 5** – Generation of an offspring characterized by Exogenic Allelism.
We crossed homozygous-plants resulted from steps 1 and 4. One plant contained the entire cassette (the entire expression cassette, EC-G+C, step 1) and the other contained only the GFP allele (expression cassette after excision, EC-G, step 4, devoid of recombinase). This should generate an offspring characterized by Exogenic Allelism, e.g., 100% of the progeny plants should include both alleles.

**Figure 13** shows gDNA analysis of 16 plants of the above described progeny. Analysis was performed as describe for figure 8. The results show that 100% of the plants contain both alleles, e.g. "…said first and second exogenes being in allelic relationship…."

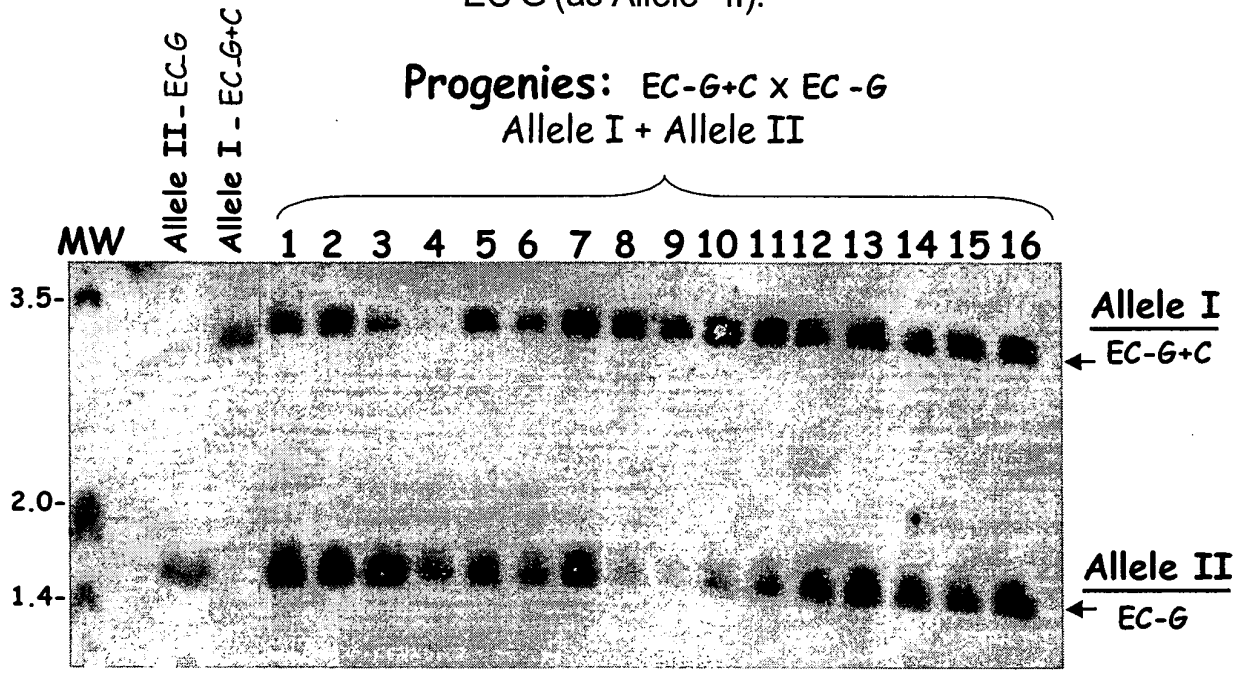
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**Figure 13**

# Claims 47d and 49d

"Crossing a plant ..... so as to generate an offspring characterized by **Exogenic Allelism"**

gDNA of progeny plants from crosses between EC-C+G (as allele I) and EC-G (as Allele II).




plants were digested with EcoRI and HindIII and probed with GFP/CFP probe

**These results demonstrate plants which carry stable exogenes in an Exogenic Allelism position**

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**Step 6** - Crossing plants resulted from step 5 (Figure 13) with WT *Arabidopsis* plants to obtain 100% segregation of alleles, "..such that the exogenes obligatory segregate to different gametes..."

Several plants from the progeny of step 5 were crossed with WT *Arabidopsis* plants and seeds were collected. Total of 62 plants of this progeny were analyzed for the presence of exogenes. **Non of the plants contained both alleles.**

**Figure 14** shows an example of gDNA analysis isolated from plants of the above described progeny.

**Table 1** summarize the statistical distribution of alleles in the plants of the above described the progeny.

**Figure 15** summarizes the concept of Exogenic Allelism, showing obligatorily segregation of exogenes in F1 plants.

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**Figure 14**

New Claims 59 and 61

"wherein the first and the second exogenes being in allelic relationship, obligatory segregate to different gametes"

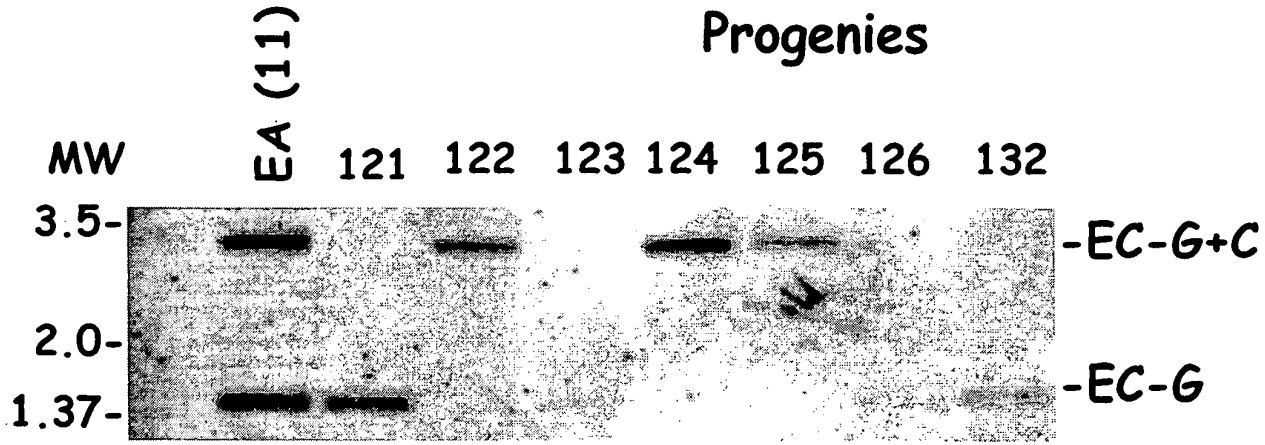


**Table 1**

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New Claims 59 and 61

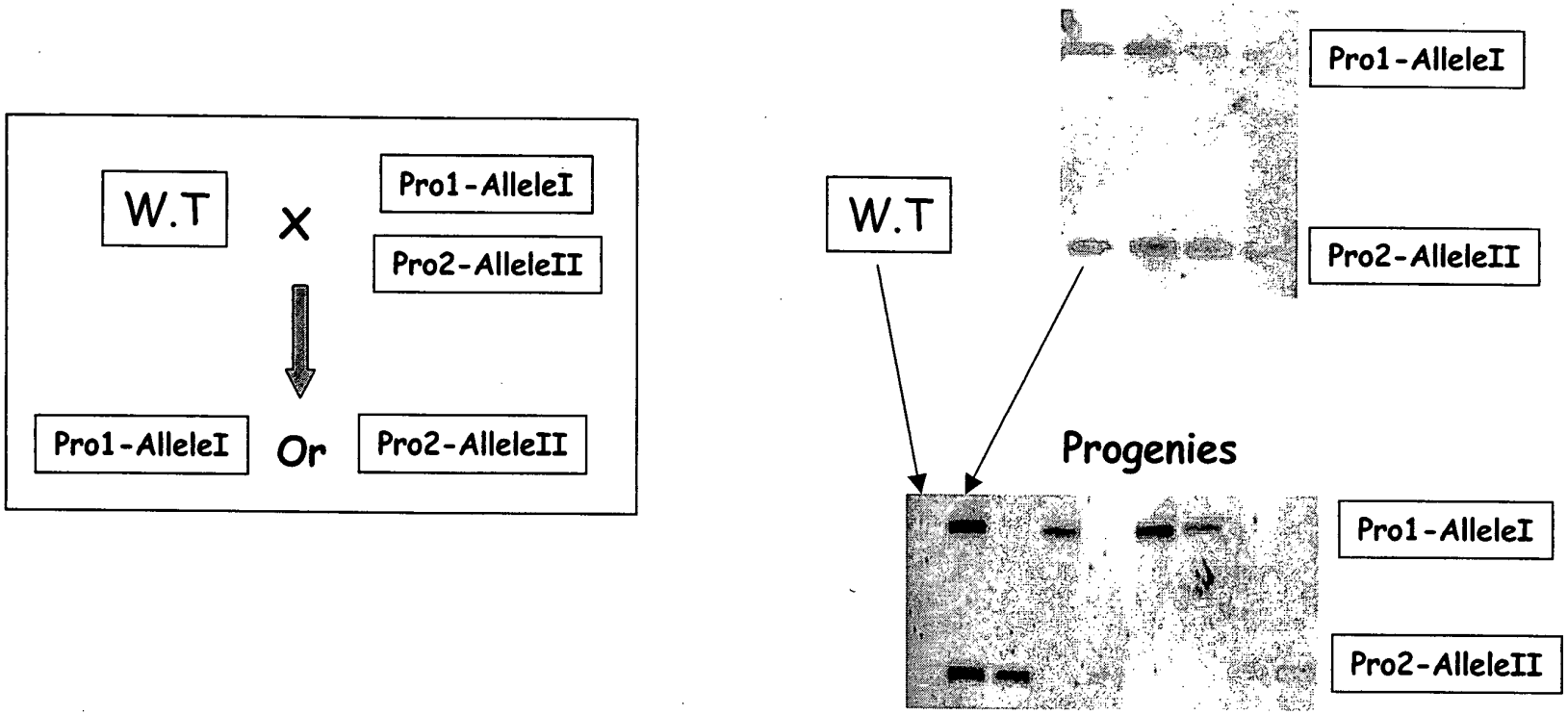
"wherein the first and the second exogenes being in allelic relationship, obligatory segregate to different gametes"

| Plant Line number | Total Plant analyzed | EC - G + C allele | EC - G allele | $X^2$ |
|---|---|---|---|---|
| 10 | 31 | 14 | 17 | 0.59 |
| 5 | 16 | 7 | 9 | 0.62 |
| 11 | 15 | 7 | 8 | 0.8 |
| Total | 62 | 28 | 34 | 0.44 |

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**Figure 15**

# Proof of concept




**These results demonstrate obligatorily segregation of exogenes in F1 plants**